

A Strip Biosensor with Guinea Green B and Fuchsin Basic Color Indicators on a Glass Nanofiber Carrier for the Cholinesterase Detection of Nerve Agents

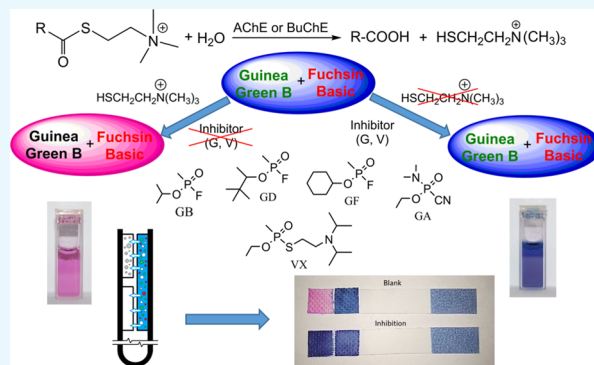
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ABSTRACT: This paper deals with the innovation of the Czech colorimetric biosensor DeteHit designed for the simple, fast, and sensitive detection of nerve agents. The innovation is based on the use of an indicator consisting of a mixture of two triphenylmethane dyes, Guinea green B and a basic fuchsin, on a glass nanofiber filter paper carrier. The advantage of this solution is the blue–red color transition, which is much more visible than the white–yellow transition of other DeteHit biosensors. The newly designed biosensor allows the users to visually detect (with the naked eye) the presence of the most significant paralytic substances (sarin, soman, cyclosarin, tabun, VX) in water at concentrations of at least 0.001 $\mu\text{g/mL}$. This biosensor design also enables one to detect these substances in air or on contaminated surfaces.



1. INTRODUCTION

Neuromuscular blocking substances have been the most important group of nerve agents since World War II. Their toxic effect is based on the inhibition of the enzyme acetylcholinesterase, which is involved in nerve transmission via the neurotransmitter acetylcholine.¹ The inhaled lethal concentration (LC_{50}) of these extremely toxic acetylcholinesterase inhibitors ranges from 15 to 70 $\text{mg}\cdot\text{min}/\text{m}^3$; the oral lethal doses (LD_{50}) of the most toxic of these substances are estimated to be 5–10 mg .^{2,3} Although they are under strict international supervision under the Chemical Weapons Convention, their use is still topical. In particular, civil wars, terrorist attacks, and particularly dangerous criminal acts pose a risk.⁴ Nerve agents are not only known as standard chemical ammunition fillers but also toxic substances with the same mechanisms of effect developed as chemical weapons in the past despite not being explicitly monitored by the Chemical Weapons Convention. An example is the compound known as Novichok, discussed in association with the Salisbury case.⁵

The analysis of the current state suggests that the detection of nerve agents/acetylcholinesterase inhibitors is still a highly topical problem. The approach to the detection process may vary. It can be based on the use of a variety of methods and procedures,⁶ but the main criteria are the aim and effectiveness of detection. In practice, this means that, in addition to advanced instrumental techniques, simple methods and technical means that require minimal servicing and are low-

cost, widely available, yet sufficiently reliable are used. A significant part of these simple methods is based on color reactions with a visual evaluation (naked eye): they can be indicative papers, test strips, detection tubes, or pocket laboratories.⁷ It appears that, due to the extreme toxicity of nerve agents with nerve-paralytic effects (on the order of 100 times that of sulfur yperite), it is necessary to use methods and biosensors with a very sensitive enzymatic (cholinesterase) reaction.⁸

The cholinesterase reaction in simple colorimetric biosensors is based on the color indication of a product with suitable substrate hydrolysis. The analyte concentration is then proportional to the degree of the enzyme inhibition and the rate of the color change. The first group of biosensors contains acetylcholine and butyrylcholine substrates, which bond to choline and the corresponding acid, which can be detected using a pH indicator. The second group, more widespread nowadays, contains the substrates acetylthiocholine or butyrylthiocholine, where thiocholine is formed instead of choline, changing the color of the redox indicators, for example, Ellman's reagent,^{9,10} 2,6-dichlorophenolindophenol¹¹ or its analogues,¹² or triphenylmethane dyes such as Guinea green B.¹³ The third group of biosensors contains chromogenic

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substrates, such as 2,6-dichlorophenolindophenyl acetate¹⁴ or indoxyl acetate,¹⁵ which hydrolyzes directly to form the dyed product.

The Czech Deteht biosensor is an example of a nerve agent biosensor, which contains acetylcholinesterase (directly from a porcine brain), acetylthiocholine, and the redox indicator Ellman's reagent.¹⁶ The Deteht biosensor provides a white-yellow color effect that can be difficult to see properly, especially under low-light conditions. The authors previously proposed some form of modification using filter paper made of glass nanofibers (as a substrate and indicator carrier), which intensifies the intensity of the resulting yellow color.¹⁷ The authors also proposed a modified biosensor with a 2,6-dichlorophenolindophenol indicator with a blue–white color transition¹⁸ and a biosensor with a Guinea green B indicator with a green–white transition.¹³

The authors of this manuscript have recently published the results of the development of a tubular detector (biosensor) of cholinesterase inhibitors where the detector uses a mixture of two triphenylmethane dyes, namely, Guinea green B and basic fuchsin, as the chromogenic indicators.¹⁹ The purpose of introducing a two-component reagent was to significantly enhance the color response of the biosensor, indicated by the blue–red transition. This paper presents the results of the experiments on the use of the mentioned two-component indicator to innovate Deteht-type biosensors for the detection of nerve agents with nerve-paralytic effects. The aim of the experiments was to verify the design of the biosensor, its stability, and its basic analytical properties, including detection limits.

2. RESULTS AND DISCUSSION

2.1. Characteristics of the Mixed Indicator. The idea of a mixed indicator as such is not new. One of the historically most well-known mixed indicators is the so-called Votoček's reagent, with a composition close to that of the mixed indicator described in this study.²⁰ This indicator contains a mixture of two triphenylmethane dyes, fuchsin and malachite green (in a 3:1 wt. ratio), which, by the action of reducing agents (sulfites), changes color more readily than the individual components of the reagent when used alone. The idea of utilizing this synergistic effect also influenced the design of a mixed indicator for the hydrolysis product of the substrate during the cholinesterase reaction.

In the experiments with mixtures of different triphenylmethane dyes with significantly different $\lambda_{\max}$ values, it was found that a mixed indicator with a characteristic color (blue) is produced under certain defined conditions (mutual ratio, pH). There is no discoloration, but it turns into a product of a markedly different color (red). The combination of GG ($\lambda_{\max} = 620$ nm) and FB ($\lambda_{\max} = 540$ nm) has been found to be the most advantageous mixture and has been previously verified by the authors in the preparation of a tubular biosensor (detection tube) for detecting cholinesterase inhibitors.¹⁹ The diagram of the function of this GG/FB indicator is shown in Figure 1. The course of the absorbance curves of the GG/FB indicator measured at $t = 0$ and $t = 10$ min is shown in Figure 2a. As shown in Figure 2b, both components of the indicator are discolored by the action of the hydrolytically released thiocholine, but this gradient is significantly greater in GG.

2.2. Effect of the Reaction Conditions. **2.2.1. Effect of the Carrier.** The condition for the good performances of the GG/FB indicator and, thus, of the biosensor itself was a carrier

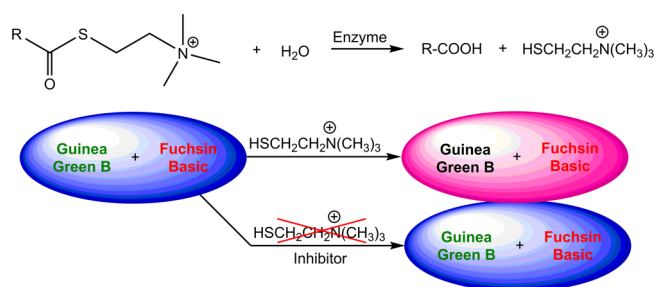


Figure 1. Schematic diagram of the GG/FB indicator function.

with a sufficient amount of both components to migrate during contact with the indicator fabric. Tests have shown that glass nanofiber filter paper is well suited for this task (Figure 3). The transport (migration) of FB is of critical importance. It has been shown that its transport is about 2 times less effective in the commonly used cellulose-based carriers than in glass nanofibers.

2.2.2. Effect of the Total Enzyme Activity. In the study of the analytical system in the liquid phase (for the blank sample), it was confirmed that the decrease in absorbance of the reaction product of the reduction of the mixed indicator (GG component measurement) is a function of the total enzyme activity (Figure 4a). Figure 4b shows that, with certain enzyme activity, the absorbance value (color intensity) corresponding to the GG reduction rate decreases with time.

2.2.3. Effect of pH. The influence of the reaction's medium pH on the function of the analytical system was investigated. Obviously, this system works the most reliably in an environment close to the physiological pH, that is, in the range of 7.0–8.0.

2.2.4. Effect of the Incubation Time. We analyzed the influence of the incubation time on the function of the analytical system in the PHY samples. This effect is illustrated in Figure 5a, which shows that the increased incubation time results in an increase in the detection sensitivity—the GG discoloration rate increases. The graphs may be interpreted to mean that the inhibition of PHY with a concentration of 0.1 mg/mL and a 5 min incubation period is approximately the same as that of PHY with a concentration of 0.2 mg/mL and 2 min incubation period (a 2-fold decrease in the concentration = 2.5-fold increase of the incubation period). The dependence of the absorbance on the PHY concentration at a constant incubation time is illustrated in Figure 5b.

2.3. Function of the Proposed Biosensor. The function of the proposed biosensor is illustrated in Figure 6a, which shows the appearance of the indicator fabric after use of the blank (change of the blue color to red) and a sample contaminated with an inhibitor (the blue color does not change). The graph in Figure 6b shows the dependence of parameter a^* on the reaction time for the blank sample. Parameter a^* corresponds to the decrease in the green color (GG) and, thus, the higher saturation of the red color of the GG/FB indicator. The blue–red transition (visually assessed) is virtually completed within 60 s.

The function of the proposed biosensor in the presence of cholinesterase inhibitors has been studied in more detail for PHY, for both lyophilized enzymes (AChE, BuChE). It was confirmed that increasing the concentration of the inhibitor decreases the green component of the dye in both cases (Figure 7). Furthermore, Figure 8, for the examples of PHY and DCP, shows that their ability to inhibit the enzyme

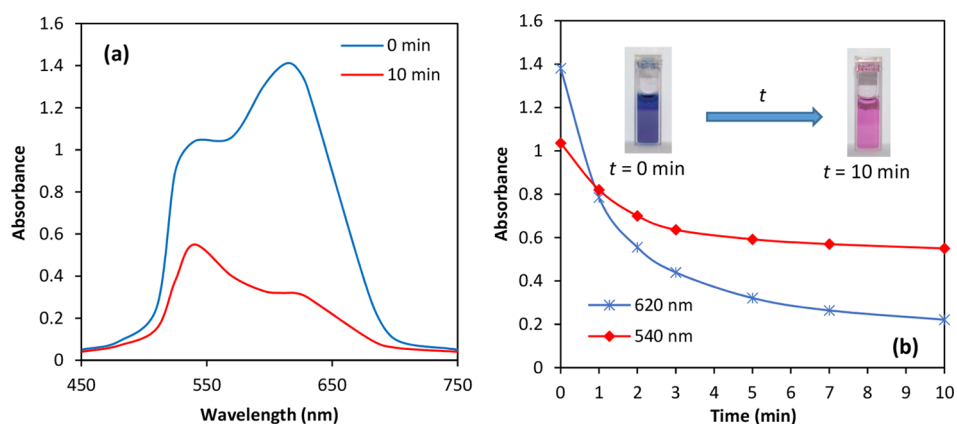


Figure 2. Characteristics of the GG/FB indicator: (a) the absorbance curves of the original indicator ($t = 0$) and after the change in color ($t = 10$ min); (b) dependence of the absorbance on time for the individual components. Photos taken by L. Matějovský.

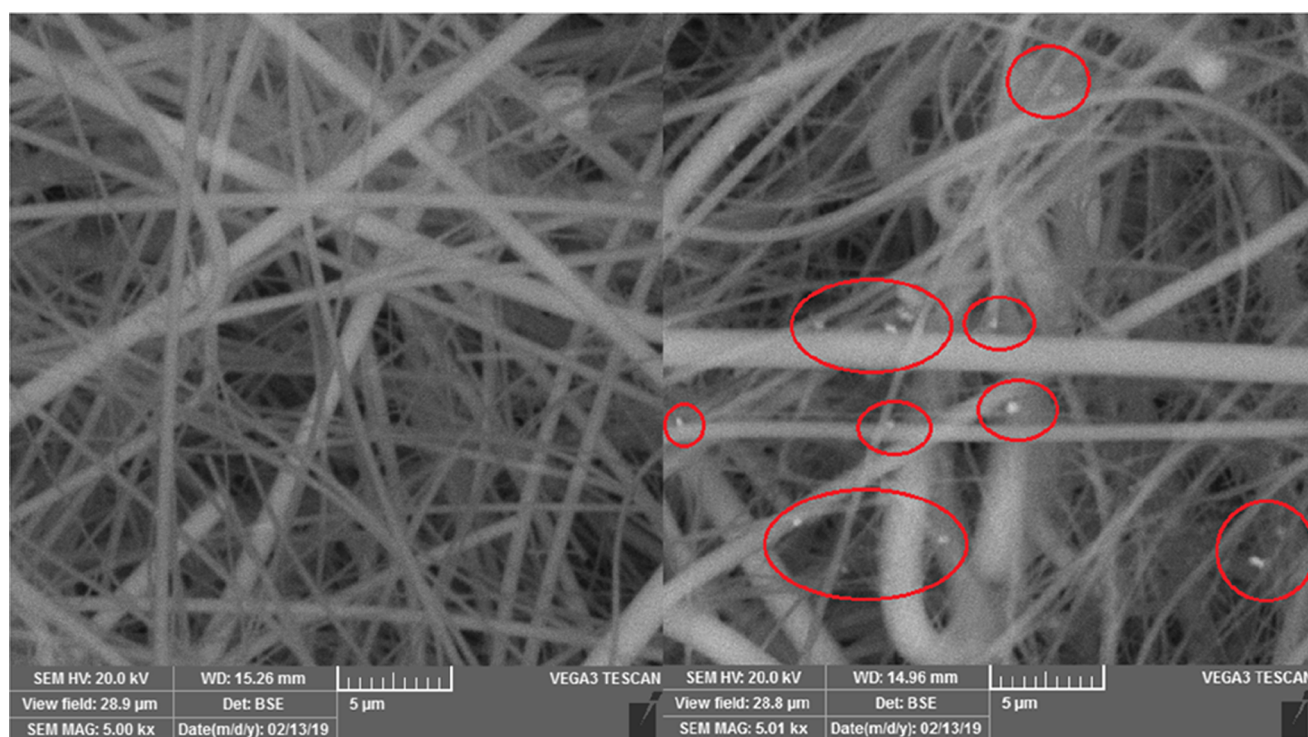


Figure 3. SEM images of the glass nanofiber filter paper before impregnation (left) and after impregnation (right) with the substrate and GG/FB indicator.

increases proportionally with an increase in concentration. However, it can be seen from the comparison of the graphs that the enzymes were variously sensitive to the used inhibitors. AChE was more sensitive to the carbamate inhibitor PHY, while BuChE was more sensitive to the organophosphate inhibitor DCP. This results from the chemical structure. Both enzymes not only have different substrate specificities but generally also exhibit partial differences between the affinities of individual inhibitors for the catalytically active centers.^{1,2,21}

2.4. Limits of Detection. The evaluation of the proposed biosensor was based on visual observation (naked eye) of the complete change in color of the indicator zone (blue–red), which depends on the concentration of the cholinesterase inhibitor present. We have come to several conclusions when studying this dependence in simulants, which are well in line with previous experimental data. As shown in Figure 9, the

BuChE biosensor was more sensitive to the presence of DCP (panel (a)), while the biosensor with AChE was more responsive to PHY (panel (b)).

The limits of detection for real nerve agents in water, determined under the same conditions and the same assessment method (Sections 4.3 and 4.5), were less than 0.001 μg/mL. As shown in Table 1, this concentration provided 33 to 57% inhibitory activity (versus 25%). The biosensor provided the best results with GF and GD; contrary to our expectations, it was more sensitive to GA than to GB. The limits of detection for all the tested nerve agents comply with the generally accepted water safety requirements during emergencies (0.012 μg/mL for the consumption of 5 L/day).²² The comparison of the limits of detection of the biosensor with standard Detehit biosensors and their previous modifications is provided in Table 2.

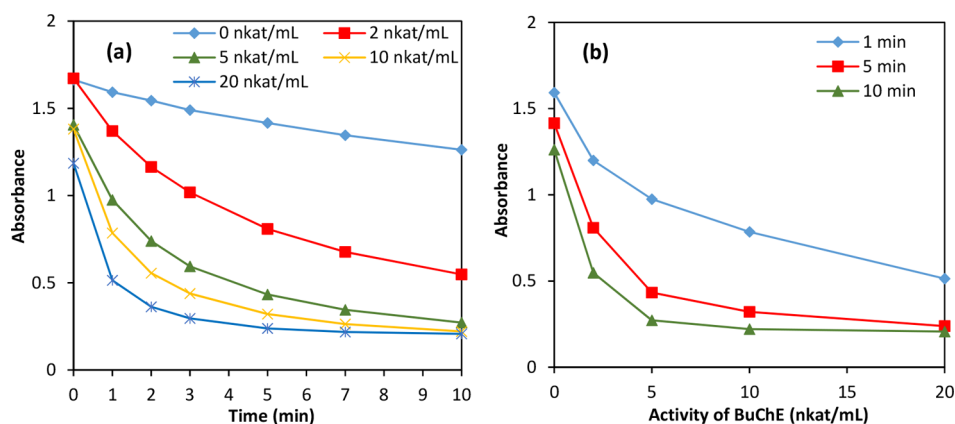


Figure 4. Interaction of absorbance, reaction time, and BuChE activity (620 nm wavelength, pH 7.5): (a) the effect of enzyme activity on the development of absorbance over time; (b) the effect of reaction time on the dependence of absorbance on enzyme activity.

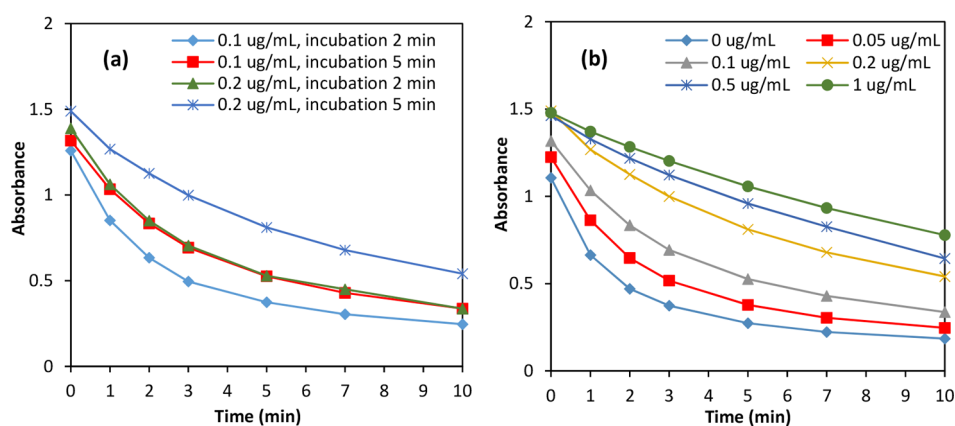


Figure 5. (a) Effect of the incubation time and PHY concentration on the stability of GG/FB indicator color (BuChE 10 nkat/mL, pH 7.5, absorbance 620 nm); (b) the effect of the PHY concentration on the color change (incubation period of 5 min).

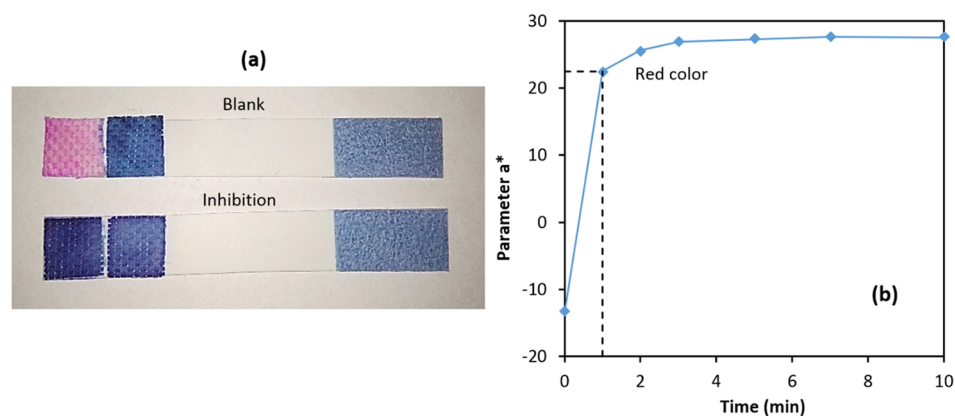


Figure 6. (a) Function of the proposed biosensor; (b) course of the change in color of the indicator fabric (blank sample). Photo taken by L. Matějovský.

For safety reasons, the detection limits have not been verified using nerve agent samples in air, but based on long-term experience with biosensors of this type, we may assume that they will be about 0.01 mg/m^{-3} and below (after a 60 s exposure); this corresponds to acute exposures at AEGL-2 (8 h) for GA, GB, GD, and GF and AEGL-3 (for 1 h) for VX.²³ The experimental results enable us to provide some partial conclusions about the possibilities of further increasing the sensitivity of the biosensor: reducing the activity of the enzyme immobilized on the fabric, prolonging the exposure

time (up to 30 min), prolonging the incubation period, or modifying the assessment method (including the objective measurement of the color changes, for example, a tristimulus colorimeter).

2.5. Stability. **2.5.1. Stability of the Immobilized Enzymes.** The resistance of the individual types of enzymes immobilized on a fabric to elevated temperatures was assessed as a part of accelerated stability tests. As shown in Figure 10, at 60°C , commercially available lyophilized enzymes exhibited greater stability than AChE obtained directly from brain tissue

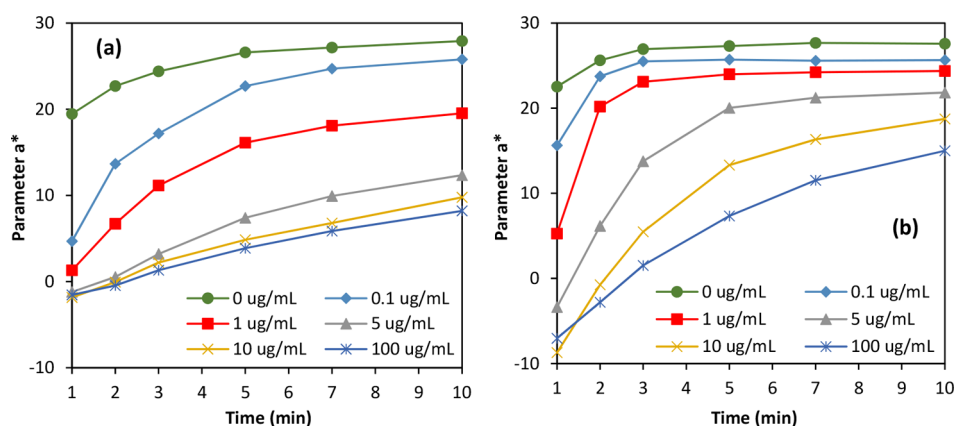


Figure 7. Effect of the PHY concentration (incubation period of 2 min) on the change in color of the indicator fabric: (a) AChE; (b) BuChE.

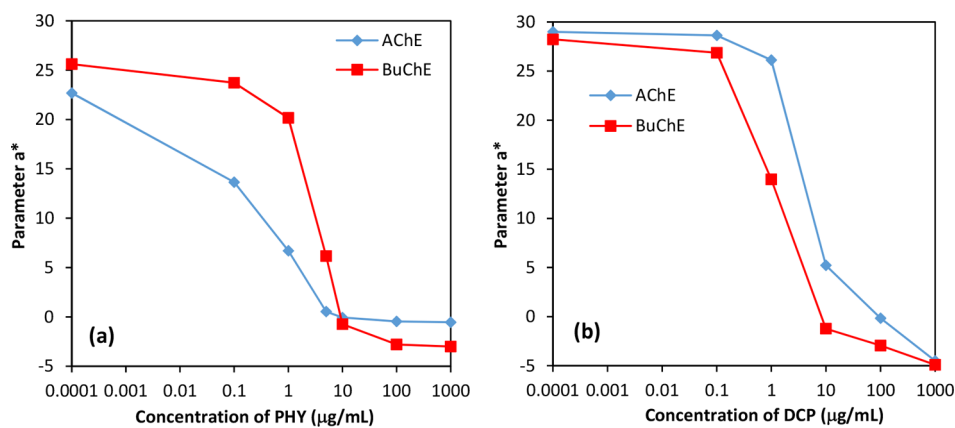


Figure 8. Effect of the inhibitor concentration on the intensity of the indicator fabric (incubation period of 2 min, measurement time 2 min): (a) PHY; (b) DCP.

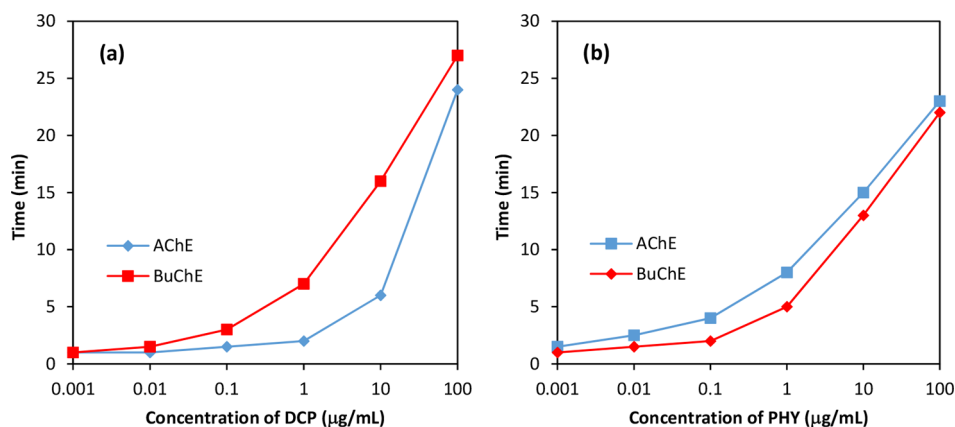


Figure 9. Effect of the inhibitor concentration on the change in the dyed indicator fabric with AChE or BuChE: (a) DCP; (b) PHY.

used in the Deteht biosensor. This difference in stability was particularly apparent in testing with a duration range of 72–240 h.

2.5.2. Stability of the GG/FB Indicator and Substrates. Accelerated stability tests were also carried out with the GG/FB indicator in combination with the substrates (ATChI, BuTChI). Both base carriers, cellulose paper and glass nanofiber paper, were impregnated with this substance (temperature 60 °C for 480 h). The initial stability assessments were performed by tristimulus colorimetric measurement of the changes in the original color of the impregnated carrier at

regular time intervals. As illustrated in the graphs in Figure 11, the glass nanofiber paper samples exhibited significantly higher stability than the cellulose paper samples. The combination of the GG/FB indicator with BuTChI was the most stable. In further experiments, it was verified that the stability can be further improved by decreasing the substrate concentration and FB while maintaining a mutual weight ratio (Figure 12a). For comparison, Figure 12b shows the course of the change in color of the paper from glass nanofibers impregnated with the substrate and GG/FB indicator at room temperature. It can be

Table 1. Biosensor Response to the Inhibitor Samples at Different Concentrations^a

sample	0.001 $\mu\text{g/mL}$		0.01 $\mu\text{g/mL}$		0.1 $\mu\text{g/mL}$	
	dyeing time (s)	I (%)	dyeing time (s)	I (%)	dyeing time (s)	I (%)
GB	90	33.3	195	69.2	>300	>80
GF	140	57.1	300	80.0	>300	>80
GD	140	57.1	220	72.7	>300	>80
GA	110	45.4	180	66.6	>300	>80
VX	120	50.0	270	77.8	>300	>80

^aBuChE-BuTChI system, 60 s incubation period.

seen that, starting at day 90, the stability was virtually unchanged or it changed minimally.

The initial stability assessment was combined with the analysis of the behavior of the glass nanofiber carrier samples where the enzyme (AChE, BuChE) was exposed to a high PHY. Some of the findings of this study are illustrated in Figure 13. Both graphs (Figure 13a,b) show that parameter b^* is virtually the same, which may be interpreted that the concentration of the original FB in the GG/FB indicator is virtually unchanged (minimal reduction occurs). Conversely, the concentration of the original GG (parameter a^*) changes significantly (reduction) depending on the testing temperature (60 °C or normal laboratory temperature). It has been confirmed that the GG/FB-impregnated glass nanofiber paper and BuTChI, in combination with BuChE, provide the best results.

3. CONCLUSIONS

The standard Deteht biosensor for the detection of nerve agents with nerve-paralytic effects improved using an indicator consisting of a mixture of two triphenylmethane dyes (GG/FB indicator). The indicator components are highly compatible and likely to have a synergic effect. The reliable performance of the indicator is ensured by a special glass nanofiber carrier. BuChE was preferred to run the enzymatic reaction together with a compatible BuTChI substrate. Although the importance of BuChE compared to AChE is not fully understood, its use has a number of practical advantages: BuChE is not inhibited by substrate excess as is typical for AChE, and the active center is wider, excels in high stability, and thermal stability after immobilization on the fabric. The biosensor allows for the rapid, simple, and robust detection of these nerve agents at concentrations less than 0.001 $\mu\text{g/mL}$, with its distinctive blue–red color change (compared to previous modifications), beneficial for visual evaluation (naked eye). The results show that the newly developed GG/FB indicator biosensor can enhance the quality of the in situ analysis of nerve agents, especially in extreme field conditions. Concerning the dual-skinned GC/FB indicator itself, it turns out that some complex

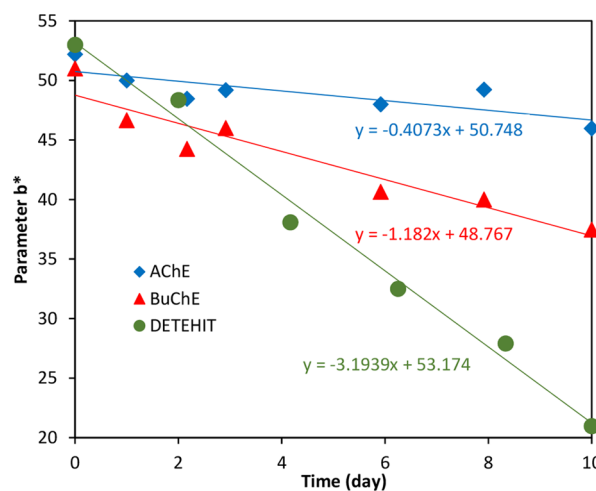


Figure 10. Stability of the immobilized enzymes on the fabric exposed to 60 °C (Ellman's reagent test – a^* characterizes the intensity of the yellow color, measured after 2 min on the tristimulus colorimeter).

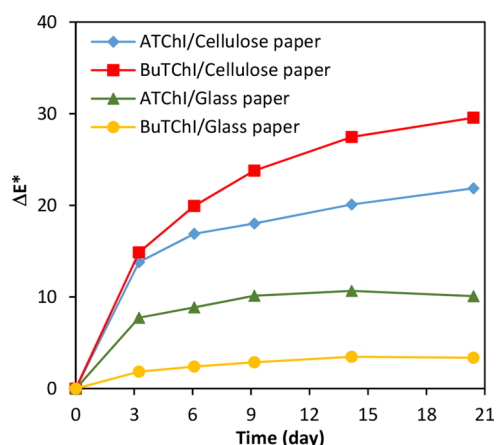


Figure 11. Stability of the cellulose paper and the glass nanofiber paper impregnated with 7 mmol/L substrate and GG/FB indicator (FB 140 mg/L) at 60 °C, based on the change of ΔE .

detection problems do not have to be solved by the time-consuming and cost-intensive synthesis of new analytical reagents but can be achieved much more efficiently by using existing indicators or mixtures thereof.

4. EXPERIMENTAL SECTION

4.1. Chemicals and Equipment. Cellulose filter paper with a specific gravity of 85 g/m² (Whatman, Kent, U.K.) and MN GF-5 glass nanofiber filter paper (Macherey-Nage, Dueren, Germany) with a specific gravity of 85 g/m² and a thickness of 0.4 mm were used as the carriers for the substrate and indicator. In addition, a white cellulose fabric with a

Table 2. Comparison of the Selected Parameters of the Standard Deteht Biosensor with Its Modifications^a

indicator	color change	enzyme	LOD in water ($\mu\text{g/mL}$)	note	ref
Ellman (Deteht)	W–Y	AChE	0.004; 0.03	GD; GB, VX	16
Ellman (new carrier)	W–Y	AChE; BuChE	0.0005; 0.005	GB; GB	17
2,6-dichlorophenolindophenol	B–W	BuChE	0.01	PHY	18
GG	G–W	BuChE	0.001	GB, GD, GF, VX	13
GG/FB proposal	B/R	BuChE	<0.01	GA, GB, GD, GF, VX	

^aW: white, Y: yellow, B: blue, G: green, R: red.

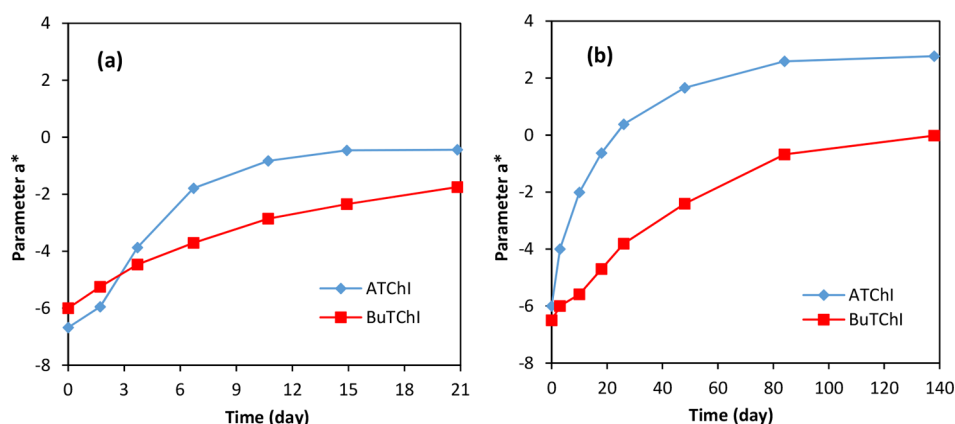


Figure 12. Stability of the cellulose paper and the glass nanofiber paper impregnated with 3.5 mmol/L substrate and GG/FB indicator (FB 60 mg/L), based on parameter a^* change: (a) 60 °C; (b) room temperature.

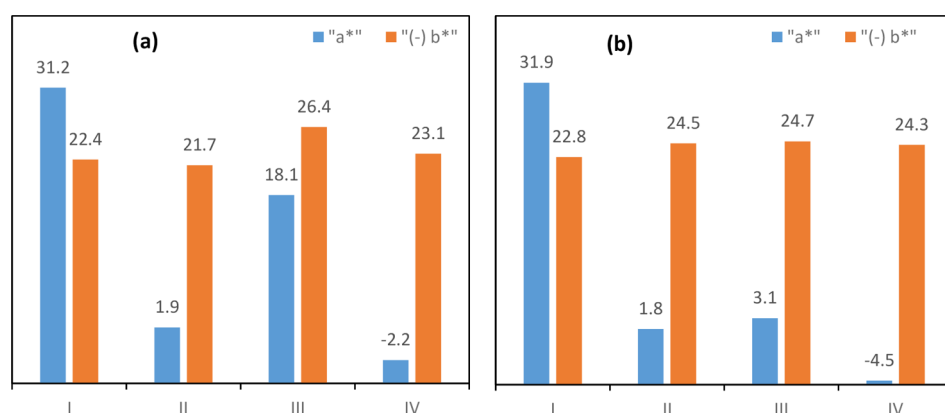


Figure 13. Performance of the glass nanofiber paper impregnated with the substrate and the GG/FB indicator after stability test at 60 °C (parameters a^* , b^*). I – freshly prepared according to the standard procedure, II – half the concentration of the substrate, after 240 h, III – half the concentration of the substrate, after 480 h, IV – half the concentration of the substrate, after 100 days. The PHY inhibitor 0.0 mg/mL, measured after 2 min. Dependency: (a) ATChI; (b) BuTChI.

specific gravity of 173 g/m² was used to immobilize the enzyme. The following chemicals were used to impregnate the filter papers: ethanol 99%, methanol 99% (both Penta, Prague, Czech Republic), redistilled water, 5,5'-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent), Fuchsin Basic (FB, parosaniline, C.I. 42500), Guinea green B (GG, Acid Green 3, C.I. 42085), acetylthiocholine iodide (min. 99%, ATChI), and butyrylthiocholine iodide (min. 99%, BuTChI) (all Sigma-Aldrich, St. Louis, MO, USA). Lyophilized enzymes butyrylcholinesterase (BuChE) and acetylcholinesterase (AChE) from horse plasma (both Sigma-Aldrich) with a declared activity of 0.1667 μ kat/mg, followed by AChE from animal brain tissue (Oritest, Prague, Czech Republic), were used to impregnate the cellulose fabric. Corn dextran (Sigma-Aldrich) and anionic surfactant C₁₂₋₁₄ alcohol 7EO (Enaspol, Velvěty, Czech Republic) were used to immobilize the enzyme to the fabric. Buffer solutions were prepared using Na₂HPO₄ and KH₂PO₄ (Sigma-Aldrich).

Due to safety and hygiene reasons, the basic biosensor function was tested with commonly used simulants, physostigmine (PHY) and diethylchlorophosphate (DCP) (both Sigma-Aldrich). The results were verified using real samples of nerve agents: sarin (GB), soman (GD), cyclosarin (GF), tabun (GA), and VX (all from the Military Research Institute, Brno, Czech Republic). The purity of nerve agents ranged between 88 and 92% as confirmed by GC–MS. The

chemical structures of the used simulants and nerve agents are shown in Figure 14.

Aquamate (Thermo Spectronic, Cambridge, U.K.) was used for the spectrophotometric measurement in the solutions. Tristimulus colorimetric measurements were performed using an LMG 173 portable colorimeter (Dr. Lange, Dusseldorf, Germany).

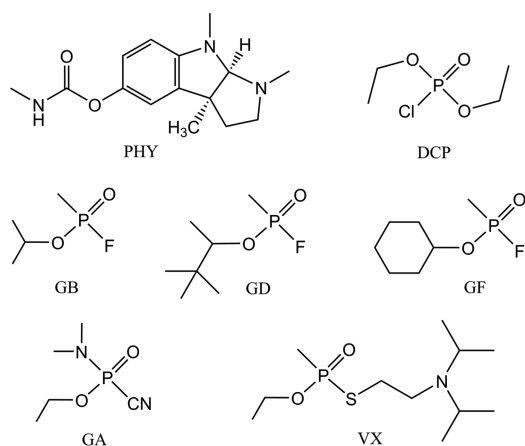


Figure 14. Chemical structures of the test substances.

4.2. Biosensor Preparation. The biosensor consisted of a plastic strip 10 cm long by 1 cm wide by 0.5 mm thick, which was provided with an indicator fabric (1 cm²) with immobilized enzymes at one end, and the fabric (1 cm²) without the enzyme was used as a control. At the opposite end, the biosensor was provided with a carrier (2 cm²) impregnated with a mixed indicator (GG/FB indicator) and substrate. The design allowed for the mechanical coupling of the opposite ends of the detector so that the two fabrics were completely covered. A schematic diagram of the construction of the biosensor including its use is shown in Figure 15.

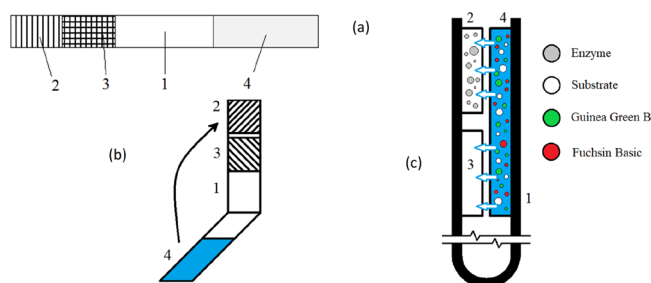


Figure 15. (a) Biosensor schematic diagram: 1 – plastic strip, 2 – fabric with immobilized enzyme, 3 – control, 4 – substrate carrier and indicator; (b) the direction of the connection of the opposite ends of the biosensor; (c) the scheme of the transport of the reagents from the paper to the fabric.

A solution containing an adequate amount of the enzyme with a total activity of 21 nkat/mL, 5% dextran, and 2% surfactant in the phosphate buffer of pH 7.5 was used to impregnate the cellulose fabric.

The solution for impregnating the cellulose paper and the glass nanofiber paper contained 60–140 mg/L FB, 1.4 g/L GG, 3.5–7 mmol/L ATChI or BuTChI, 60% ethanol (v/v), 30% methanol (v/v), and 10% water (v/v).

4.3. Method of the Detector Function Testing. The biosensor function was tested by wetting the indicator fabric in water (blank) or in an aqueous solution with PHY at concentrations of 0.001–100 µg/mL. After the exposure, the fabric was allowed to rest for 60 s for the enzyme incubation. Afterward, the opposite ends of the biosensor were connected together and pressed firmly against each other for 30 s to allow sufficient transition of the GG/FB indicator and substrate to the indicator fabric. The change in color of the indicator fabric with the immobilized enzyme was evaluated both visually (the naked eye) and by objective tristimulus colorimeter measurements at regular time intervals for 10 min.

Tristimulus colorimetry is a type of reflective colorimetry (spectrophotometry) based on the CIE- $L^*a^*b^*$ color system. In this system, L^* represents the neutral axis of brightness, a^* is the chromatic green-red axis ($+a^*$ red, $-a^*$ green), and b^* is the chromatic blue-yellow axis ($+b^*$ yellow, $-b^*$ blue). In practice, ΔE , the color difference, is also used, which is defined by the equation

$$\Delta E = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \quad (1)$$

where ΔL^* , Δa^* , and Δb^* are the differences between the individual L^* , a^* , and b^* values of the standard and controlled colors. The values on the a^* , b^* , and ΔE axes were used as the analytical signals in the study.

4.4. Method of Stability Testing. The stability of the immobilized enzymes on the fabric and the stability of the GG/FB indicator and the substrate on the carrier were quickly tested in an oven at 60 °C. The performance of the enzyme fabric was tested at 240 h time intervals with the detection paper impregnated with a solution of 1.6 g/L Ellman's reagent and 6 mmol/L substrate (ATChI or BuTChI). The fabric sample was wetted with water and incubated for 1 min, and a detection paper was put on the fabric for 30 s. If the enzyme was functional, a yellow color was produced on the fabric, the intensity of which was measured in 2 min using the tristimulus colorimetry method.

The stability of the GG/FB indicator and the substrate was tested at regular intervals for 480 h by measuring the change in carrier surface color (rate of aging) by tristimulus colorimetry. After 240 and 480 h, we tested the transport of the GG/FB indicator and the substrate to the fabric, when the enzyme was inhibited by the PHY solution, with a concentration of 100 µg/mL.

4.5. Method of Determining the Detection Limits. The detection limits were determined based on the inhibitory activity (I) as a function of the nerve agent's concentration. The inhibitory activity was calculated using the formula

$$I (\%) = (1 - T_0/T) \cdot 100 \quad (2)$$

where T_0 is the time of the color change in seconds corresponding to the blank ($T_0 = 60$ s) and T is the time in seconds corresponding to the color change in the presence of the inhibitor. In view of the practical use of the biosensor, especially in difficult field conditions, the inhibitory activity was determined visually (naked eye). The detection limit corresponded to an inhibitor concentration of at least 25% of the inhibitory activity ($I = \geq 25\%$).

4.6. Spectrophotometric Measurement Procedures. The basic preliminary studies of the GG/FB indicator function were performed in solutions using spectrophotometry. An aqueous solution of BuChE with an enzyme activity of 45 nkat/mL, a solution of the BuTChI substrate in ethanol with a concentration of 16 mg/mL, and a mixed indicator solution were used in the study. The GG/FB indicator was prepared by dissolving the individual components in 60% ethanol so that the FB content was 0.4 mg/mL and GG 2.6 mg/mL.

A phosphate buffer with pH 6.5–9.0 with the enzyme solution was mixed in the tube to give a final volume of 2 mL, and the enzyme activity after the addition of the GG/FB indicator and substrate was at the level of 2.0–20 nkat/mL. Then 0.2 mL of the substrate solution and 0.025 mL of the GG/FB indicator solution were added to the tube. The absorbance values at 540 and 620 nm were measured at regular intervals for 10 min after mixing.

In the inhibitor test, a phosphate buffer with pH 7.5 was mixed with the enzyme solution to get a total volume of 2 mL with an enzyme activity of 10 nkat/mL. A solution of PHY in ethanol was added in an amount so that the final concentration was in the range of 0.05–1 µg/mL. After incubation (2–5 min), 0.2 mL of the substrate solution and 0.025 mL of the GG/FB indicator solution were added. The absorbance was then measured in the same way as for the blank.

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Notes

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■ ABBREVIATIONS

AChE, acetylcholinesterase; ATChI, acetylthiocholine iodide; BuChE, butyrylcholinesterase; BuTChI, butyrylthiocholine iodide; DCP, diethylchlorophosphate; FB, fuchsin basic; GA, tabun; GB, sarin; GD, soman; GF, cyclosarin; GG, Guinea green B; PHY, physostigmine

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